# The embryo-sac of Alisma Plantago.[1]

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WITH PLATES IX AND X.

The embryo-sac of angiosperms still presents an inviting field for research. There seem to be many variations in the general processes which occur in it, and most of the observations on the finer structure need confirmation. Especially in regard to the real meaning of the conjugation of the polar nuclei, and what is represented by the antipodal cells, does there still seem to be much obscurity. It was for the purpose of making a preliminary study with a view for further investigation later, that the following work on the embryo-sac of Alisma was undertaken.

The embryo-sac of *Butomus umbellatus* L. was described by Vesque[2] in 1878, and later by H. Marshall Ward,[3] who also made a few observations on *Alisma Plantago* L., which agreed in general with those on Butomus. The development of the embryo of Alisma has become well known through the investigations of Hanstein.[4] Guignard[5] studied the minute processes which occur in the embryo-sac of Lilium Martagon and described the conjugation of the centrospheres of the sperm nucleus and oosphere. He observed the same process during the union of the two polar nuclei. These observations have hitherto not been confirmed.

My material was collected on July 30, 1895, and killed in a chrom-acetic acid solution: acetic acid 0.7 per cent., chromic acid 0.3 per cent., water 99 per cent. All the sections were made by imbedding in paraffin and staining on the slide. The principal stains used were anilin-safranin and acid fuchsin. The work was carried on under the direction of Professor

[1] Contribution from the Botanical Laboratory of the University of Michigan.

[2] Développement du sac embryonnaire des phanérogames angiospermes. Ann. des Sci. Nat. Bot. VI. **6**: 237–285. 1878.

[3] Contributions to our knowledge of the embryo-sac in angiosperms. Jour. Linn. Soc. Bot. **17**: 519–546. 1880.

[4] Die Entwicklung des Keimes der Monokotylen und Dikotylen. Botanische Abhandlungen aus dem Gebiet der Morphologie und Physiologie **1**: —. 1870. [Bonn.]

[5] Nouvelles Etudes sur la fécondation, etc. Ann. des Sci. Nat. Bot. VII. **14**: 163–296. 1891.

F. C. Newcombe, to whom I here express my sincere thanks for kind assistance rendered in various ways.

### Development of the embryo-sac.

The young ovaries arise as protuberances around the edge of the flattened receptacle, and soon there appears in the interior of each one a schizogenetic cleft on the inner side of which the nucellus is formed. This cleft increases in size quite rapidly by enlargement and division of the cells of its walls, thus making room for the developing nucellus. When the nucellus has obtained some considerable size, there can be seen in it a large hypodermal cell which appears to be the archesporium. No division of this cell into two was observed, but at a later stage the large macrospore shows the remains of a former cell at its micropylar end (fig 1.) which is the tapetal cell. The ovule soon becomes anatropous. The integuments are two in number, but on the side of the funiculus the outer one is generally not developed. As the embryo-sac increases in size the ordinary divisions of its nucleus take place. First it divides into two, one of the daughter nuclei passing to the upper and the other to the lower end of the sac, after which each of these, by two successive divisions, produces four nuclei, thus making the typical eight-celled embryo-sac (fig. 2). Often only two nuclei could be distinguished at the base of the embryo-sac. But as it is quite narrow at this end the missing ones may have been in the adjacent sections, and there indistinguishable from the nuclei of the surrounding tissue.

All the nuclei except the three antipodal nuclei are comparatively large in size and each one contains usually one large nucleolus, but sometimes two (fig. 2). The two synergidæ lie side by side. They are surrounded by granular cell walls, and their nuclei are either spherical or ellipsoidal. They stretch across the entire upper end of the sac, and just beneath them the oosphere is suspended in a dense mass of cytoplasm. Its nucleus is usually ellipsoidal in shape. Beyond the oosphere and lying free in the cytoplasm is the upper polar nucleus. Its centrospheres usually lie on the under side, though in a few cases they were on the side toward the oosphere. The cells in the antipodal region simulate the arrangement in the egg-apparatus. There are two small nuclei lying at the base; and beyond them is the third antipodal nucleus. This nucleus always stains a very dense red with anilin-safranin and its centrospheres are usually very prominent. It would by its peculiar appearance suggest that it may be the homologue of the oosphere. These three nuclei are not surrounded by any definite cell-walls, but the cytoplasm in which they are imbedded is rather dense. Immediately beyond the antipodal cells is the lower polar nucleus. This is much the largest nucleus in the antipodal region, and it contains usually one large nucleolus. Its centrospheres lie on the upper side, toward the upper polar nucleus.

**Conjugation of the polar nuclei and their centrospheres.**

The two small granules lying on one side of the resting nucleus are now generally called controspheres by English writers, and this term is here employed as the most appropriate. The dense centre is appropriately designated by the usual term "centrosome," while "attraction-sphere" will be used for the hyaline layer surrounding this.

I might here state that all my observations on these bodies made heretofore[6] have been more fully confirmed during the present investigation. The centrosphere is composed of a dense central body, which appears to have a granular structure, and an outer hyaline layer, composed of a highly refractive substance, which takes little or no stain, but under favorable circumstances shows a dẹlicate radiate structure. The whole structure is limited from the surrounding cytoplasm by a definite granular layer, which is easily distinguished and may appropriately be called a membrane. The centrospheres in the embryo-sac are much more prominent than in the surrounding tissues, and in this respect they agree with the nuclei.

As stated above, when the two polar nuclei begin to travel toward each other, they have their centrospheres so situated that they precede the nuclei; however, in a few cases, at the very beginning they were seen on the opposite side. In my preparations I was able to find all stages of the conjugation of the polar nuclei, until their complete union to form the nucleus of the embryo-sac, or definitive nucleus (figs. 3, 4, 5, 6). My observations agree in general with those of Guignard on the conjugation of the polar nuclei in Lilium Martagon.

[6] The nature and distribution of attraction-spheres and centrosomes in vegetable cells. Bot. Gaz., **19**: 445-459. 1894.

Of the two conjugating nuclei, the one from the upper end of the sac is nearly always the larger. When the two nuclei approach each other, the centrospheres join two and two, and the two couples then separate so as to permit the two nuclei to come in contact (fig. 3). While the two nuclei are fusing, the attraction-spheres gradually unite and in them can be distinguished the two controsomes lying very close together (figs. 4, 5, 6). When the two nuclei have nearly fused, the centrosomes are in contact and are also uniting (fig. 6). The fusion of the two nuclei takes place by a gradual interchange of their contents. The chromatin does not seem to unite as definite chromosomes, but there appears to be a gradual intermingling of the individual particles from which a new chromatin network is built up. The nucleoli of the two nuclei appear distinct even to an advanced stage of the conjugation, but later there appears but one, in most cases, in the definitive nucleus, showing that there has been a union of the material of the two nucleoli, or the formation of a new one from nucleolar matter derived from the old ones (fig. 7). But the intermediate processes were not observed.

The endosperm is not very abundant when the embryo is completely developed. It appears to be formed entirely from the definitive nucleus. The first division of this nucleus generally precedes the first division of the oospore (fig. 19). These nuclei continue to divide and spread out through the embryo-sac as it increases in size. In one case I observed four endosperm cells at the time when the first division of the oospore was in the close daughter-skein stage (fig. 8).

**Phenomena of fertilization.**

The young pollen grain has two nuclei, a large one, which never stains very dark with anilin-safranin, and a smaller one which takes an intense dark red stain (fig. 9). Subsequently this small nucleus divides again (figs. 10, 11, 12), thus leaving the mature grain with one large nucleus and two small ones. These small nuclei always stain a very intensive red with anilin-safranin. When the pollen grain falls on the stigma and germinates, its tube passes through the style and thence down through the tissue on the inner side of the ovary. At the base of this the tissue is so arranged that the tube readily finds its way toward the base of the funiculus, where it comes into the free cavity of the ovary. Here it is generally much contorted before reaching the micropyle, through the middle of which it finally passes to the top of the embryo-sac. Just before the entrance of the pollen tube into the micropyle, the two synergidæ, as stated before, lie at the summit of the embryo-sac, with the oosphere suspended below. The tube in passing through the micropyle is considerably constricted, but when it reaches the embryo-sac it increases appreciably in diameter (fig. 14). On reaching the apex of the sac, the pollen-tube does not grow directly toward the oosphere, but always passes down on one side near the wall of the embryo-sac encountering the nucleus of one of the synergidæ on its passage, which disappears at this time. The tube passes by the nucleus and seems to be nourished by its contents. At a certain stage the outline of the nucleus and its nucleolus can still be distinguished, but it takes little or no stain (fig. 15).

The nucleus of the other synergida persists for a long time, at least until the proembryo is composed of several cells. The pollen-tube after entering the embryo-sac takes a very dark-red stain and in it can be distinguished two small nuclei, which stain exactly like those in the mature pollen-grain. The deep red staining of the tube is perhaps due to the nourishment which it receives from the disintegrating synergida. The upper nucleus of the pollen-tube always appears spindle-shaped or much elongated (figs. 7, 13, 14, 16). The lower one is more spherical and is preceded by two centrospheres (figs. 7, 13, 16). These centrospheres are usually very distinct, because of the clear color of the attraction-spheres in contrast to the dark red stain of the surrounding material. The upper nucleus remains in the tube after fertilization is accomplished (figs. 18, 19). The pollen-tube usually curves toward the oosphere before the sperm nucleus leaves it (fig. 17).

In the meantime changes are taking place in the oosphere. The centrospheres lie on the side toward the sperm nucleus (figs. 13, 16, 17). They also appear to travel toward the sperm nucleus, since at this stage they are farther from the nucleus than usually (figs. 13, 17). The nucleus of the oosphere is no longer symmetrical in outline as in the earlier stages, but is drawn out on the side toward its centrospheres into a considerable bulge (figs. 13, 16, 17). Thus it will be seen that all the preliminary stages in the approach of the two sexual nuclei indicate a conjugation of the centrospheres, or what has been called a "quadrille of the centres."

I made hundreds of sections in the vain attempt to follow out the stages of conjugation of the male and female pronuclei, but my material proved to be very unfavorable. I succeeded in finding but one instance of actual conjugation (fig. 18). In this section the sperm nucleus had left the pollen-tube and was in contact with the egg nucleus. No centrospheres were seen. They were very likely lying just above and beneath the line of contact and were thus invisible. However, the positions of the four centrospheres immediately before conjugation indicate the same kind of action as was observed for the two polar nuclei.

Recently, several American zoologists have attempted to investigate the phenomena connected with the conjugation of the male and female pronuclei. Wheeler,[7] in studying the fertilized egg of *Mysostoma glabrum*, concludes that the archoplasm and centrosomes of the segmentation nucleus come entirely from the female pronucleus. He found no trace of such structures in the male pronucleus. Hence he thinks there can be no "quadrille of the centers." Wilson and Mathews[8] in their investigation on the echinoderm egg also tend to disprove the "quadrille of the centers."

Mead[9] studied maturation and fecundation in Chaetopterus pergamentaceus. He traces the archoplasm and centrosomes of the segmentation nucleus from the male pronucleus, and states that these structures disappear from the female pronucleus after the extrusion of the second polar body. Thus he can also find no "quadrille of the centers."

It appears that there is not so much contradiction in the observations as in the conclusions drawn. And it should be borne in mind that mere negative results of observation prove or disprove nothing. As Strasburger has indicated in his "Neue Untersuchungen," in regard to the invisibility of the nucleus in the ripe pollen-grain of many dicotyledons when treated with certain nuclear stains, the invisibility of the nucleus in such cases must not be interpreted as indicating the absence of a nucleus, but much rather that the methods of

[7]The behavior of the centrosomes in the fertilized egg of *Mysostoma glabrum* Leuckart. Jour. Morph. **10**: 305. 1895.

[8]Maturation, fertilization, and polarity in the echinoderm egg. New light on the quadrille of the centers. Jour. Morph. **10**: 319. 1895.

[9]Some observations on maturation and fecundation in *Chaetopterus pergamentaceus* Cuvier. Jour. Morph. **10**: 313. 1895.

preparation and staining were deficient. And subsequent developments prove the wisdom of the caution. When we compare the size of an ordinary nucleus with a centrosphere it will be seen that the suggestion in the present case becomes one of great importance.

No division of nuclei was observed in the pollen-tube. The two small nuclei in the tip of the tube, after it has entered the embryo-sac, are about the same size and take the same stain as those in the pollen-grain. These are the two sperm nuclei which come from the generative nucleus. In this case the division of the generative nucleus occurs in the pollen-grain, while more commonly it does not take place until the tube is entering the embryo-sac, as shown by Guignard and Strasburger. Strasburger has found[10] that this early division of the generative nucleus occurs in many monocotyledons, and dicotyledons. Whether the two sperm nuclei are of the same nature or whether only one of them has the power of fertilizing the oosphere, I could not determine. The appearance of the two nuclei after entering the embryo-sac would indicate that only one was a true sperm nucleus. The fate of the vegetative nucleus was not discovered, but no trace of it was seen after the pollen-tube had entered the embryo-sac.

## The early development of the embryo.

It was not my intention to study the development of the embryo, but while making observations to determine the length of time that the nucleus of the remaining synergida persisted, I found that the development of the proembryo, as presented in my sections, did not agree with the statements regarding Alisma in the text books.[11] After the union of the male and female nuclei, the resulting nucleus divides in a direction at right angles to the long axis of the embryo-sac (fig. 8). The upper cell with its nucleus immediately begins to enlarge, the nucleus taking a central position (fig. 20). The lower cell increases considerably in length, the lower end becoming somewhat rounded and swollen and containing the lower nucleus. This nucleus now divides again in the same direction as the preceding division, making three nuclei for

[10] Neue Untersuchungen, etc. 8.

[11] See Goebel's Outlines of Classification and Morphology, English edition, 399. 1887; and Sachs' Text-Book of Botany, second Engl. edition, 589. 1882.

the proembryo (fig. 21). At this stage the nucleus of the one synergida still survives but it shows signs of disintegration. It disappears after this stage and its contents very likely go to nourish the enlarging nucleus of the suspensor cell. The next division is again in the lowest of the three cells, in a transverse direction (fig. 22). This gives the first four cells of the proembryo. These divisions are absolutely certain, for they were traced out through stages in which the nuclei were in the close daughter-skein, which leaves no doubt as to the origin of the different nuclei in the series. The next division which occurs is in the lowest of the four cells. This now divides in a longitudinal direction (fig. 23). The development was traced no farther. But the course described above was confirmed by numerous examples. Thus the early development of the proembryo of Alisma either presents variations, or the descriptions given by Hanstein and Famintzin are incorrect. At a later stage, three suspensor cells could still be seen (fig. 24), but whether these were the same as those seen in fig. 23, or represented cells which originated by subsequent divisions, I did not determine.

## Summary.


The results of the investigation may be summed up as follows:

1. The development of the embryo-sac of Alisma Plantago represents nothing unusual, the fully matured sac having the usual eight nuclei.

2. During the conjugation of the two polar nuclei, the four centrospheres conjugate by couples, resulting in the formation of two new ones for the definitive nucleus.

3. The endosperm is not abundant and comes entirely from the division of the definitive nucleus, the antipodal cells showing no division or fragmentation.

4. The division of the generative nucleus of the pollen occurs in the pollen grain.

5. Both of the sperm nuclei enter with the pollen tube into the embryo-sac, but only the lower one takes part in the act of fertilization, the other one remaining in the tube.

6. The nucleus of one of the synergidæ is entirely absorbed when in contact with the pollen tube. The other one is later also dissolved, its substance being used probably to nourish the large nucleus of the suspensor cell.

7. The centrospheres of the lower sperm nucleus precede it as it approaches the oosphere.

8. The nucleus of the oosphere becomes bulged out on the side nearest the sperm nucleus, and its centrospheres being situated immediately opposite this bulge, travel slightly toward the approaching male nucleus.

9. All the stages preliminary to the conjugation of the male and female nuclei are favorable to, and indicate a conjugation of their centrospheres, at the time of impregnation of the oosphere.

10. After the first division of the oospore, in the development of the proembryo, the three succeeding divisions take place each time in the outermost cell, the first three divisions being transverse to the long axis of the embryo-sac, the fourth one longitudinal.

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## Explanation of Plates IX and X.

Fig. 1. A young nucellus with macrospore and remains of the tapetal cell at the upper end.

Fig. 2. Mature embryo-sac with eight nuclei.

Fig. 3. Two polar nuclei just before conjugation, showing the centrospheres joining in couples.

Fig. 4. Conjugation of polar nuclei; the attraction-spheres have fused; the centrosomes lie close together in the united attraction-spheres.

Fig. 5. Conjugation of polar nuclei; the larger nucleus is from the upper end of the embryo-sac.

Fig. 6. Advanced stage of conjugation of polar nuclei; the two couples of centrosomes are fusing.

Fig. 7. Embryo-sac, showing one of the synergidæ, the oosphere, the definitive nucleus of the embryo-sac, the three antipodal nuclei, and the pollen-tube containing two nuclei. The lower male nucleus is preceded by two prominent centrospheres.

Fig. 8. Embryo-sac with oospore dividing, and with endosperm cells.

Fig. 9. Pollen-grain with a large and a small nucleus.

Fig. 10. Pollen-grain, showing the small nucleus in stage of division.

Fig. 11. Pollen-grain with the division of the generative nucleus farther advanced.

Fig. 12. Mature pollen-grain with one large and two small nuclei.

Fig. 13. Upper end of the embryo-sac, showing the appearance of the conjugating sperm-nucleus after leaving the pollen-tube. Below this sperm-nucleus is the oosphere, while at the apex of the sac is the remaining synergida, and at the left of the latter is the second sperm-nucleus of the pollen-tube.

Fig. 14. Outer end of the ovule, showing the pollen-tube passing through the micropyle and into the embryo-sac.

Fig. 15. Upper end of the embryo-sac, with the pollen-tube absorbing the contents of the nucleus of one of the synergidæ, the latter being but lightly shaded.

Fig. 16. Embryo-sac, showing the two centrospheres of the oosphere and those of the lower sperm-nucleus in their usual position at this stage.

Fig. 17. Upper end of the embryo-sac; the oosphere is bulged out on the side toward the pollen-tube; the centrospheres lie some distance above this protuberance.

Fig. 18. Conjugation of the nucleus from the pollen-tube with the oosphere. Above to the right is the other sperm-nucleus, and to the left the remaining synergida.

Fig. 19. Embryo-sac. The upper nucleus of the pollen-tube still persists, as does also one synergida; while the oospore is in the segmented-skein stage, and the definitive nucleus of the embryo-sac has nearly completed division.

Fig. 20. First two cells resulting from the division of the oospore. The nucleus from one of the synergidæ appears at one side of the upper cell.

Fig. 21. Proembryo with three cells. The upper suspensor cell with its nucleus is becoming very much enlarged. In its upper end appears the nucleus of one of the synergidæ.

Fig. 22. Proembryo with four cells. Two endosperm nuclei appear free in the embryo-sac.

Fig. 23. Proembryo with five cells. The three nuclei in the embryo-sac are endosperm.

Fig. 24. More advanced stage of the embryo, showing three suspensor cells.